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(54) Title: EXPRESSION CONTROL POLYNUCLEOTIDES

(57) Abstract

There is provided an expression control polynucleotide of an invertase gene, which may be operably limited to a heterologous polynucleotide. Optionally the expression control polynucleotide and heterologous polynucleotide construct is transfected into host cells or organisms. Preferably the construct is used to produce a transgenic plant and the expression control polynucleotide is pollen cell–specific. A suitable expression control polynucleotide is as set out in SEQ ID No 1, especially nucleotides 3430–5349 thereof. Desirably the protein expressed by the heterologous polynucleotide causes male sterility in the transgenic plants.

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"Expression Control Polynucleotides"

This invention relates to the fields of plant biotechnology and plant genetic engineering. In particular it relates to transgenic plant production and tissue-specific expression of introduced gene sequences in pollen cells.

A promoter is a non-coding nucleotide sequence which controls the transcription of an adjacent nucleotide sequence. A number of promoters have been isolated from a wide variety of sources, including plants. In certain applications it is desirable to genetically engineer a construct which comprises a promoter operatively linked to a heterologous nucleotide sequence such that the promoter controls expression of the heterologous sequence in the host cell transformed with that construct. Where the promoter is only active in particular tissue types expression of the heterologous sequence is restricted accordingly and this may be especially desirable in some circumstances.

A number of plant-derived promoters have been isolated which activate expression of their companion nucleotide sequences only in pollen cells. Use of these pollen

cell-specific promoters to activate genes encoding heterologous proteins has also been described [see CA 2021643] and may lead to the production of proteins not normally present in pollen cells. Such an approach may allow the expression of heterologous genes which encode for proteins able to render the plant male-sterile by ablation of pollen cells (for example if the proteins are toxic to the pollen cell) or to drive the production of antisense RNAs which interfere with the normal processes of pollen cell metabolism. Pollen cell-specific promoters can further be used to drive expression of proteins that are toxic to insects or other pests which consume pollen. These promoters can also be used to activate the expression of genes encoding proteins which will enhance the nutritional value of pollen.

However, the number of pollen cell-specific promoters which have been well characterised is limited and different promoters exhibit a range of activities which cannot be predicted *a priori* and are difficult to quantify. The activity of a promoter isolated from one species of plant may also differ when the promoter is utilised in an heterologous species - such differences may be both in the tissue specificity and strength of the promoter and are more likely to occur with greater taxonomic distance between plant species. In addition different promoters may be required to control expression of multiple genes since a gene silencing effect can occur if duplicate copies of the same promoter are used. The choice of promoter is therefore limited and has to be experimentally verified in the system under study.

According to the present invention there is provided an invertase gene expression control polynucleotide, a

derivative, a functional equivalent, or a part thereof, which is pollen cell-specific.

By "pollen cell-specific" we mean that the expression control polynucleotide exhibits a distinct level of activity (or lack of activity) in pollen cells (ie in material ranging from developing pollen grain through to material derived from pollen) compared to the other tissue types of the transformed plant.

By "expression control polynucleotide" we mean any polynucleotide which is capable of affecting the expression of a gene. The term is intended to include promoters, enhancers and suppressors.

By "functional equivalent" we mean any variation of the expression control polynucleotide which exhibits substantially the same functional properties of the original polynucleotide.

By "derivative" we mean a modified version of the expression control polynucleotide which exhibits substantial sequence homology to the original polynucleotide, for example which include nucleotide substitutions which have no effect on biological function.

By "part" we mean a deleted version of the expression control polynucleotide, which comprises at least a substantial portion of the original polynucleotide (for example at least 50% of said polynucleotide).

The preferred type of expression control polynucleotide is a promoter.

The invertase gene promoter is preferably derived from

a dicotyledon, such as potato.

The expression control polynucleotide of the invention may comprise double- or single-stranded DNA or RNA.

The invention also provides the use of the expression control polynucleotide described above to control expression of heterologous sequences. Optionally the expression control polynucleotide is used to drive pollen cell-specific expression of protein-encoding heterologous genes in plants eg monocotyledons or dicotyledons. Use of the expression control polynucleotide in this way in dicotyledons is preferred.

The invention also provides a recombinant expression control polynucleotide comprising at least a part of a pollen cell-specific expression control polynucleotide as described above. The recombinant expression control polynucleotide of the invention is capable of specific expression of a heterologous sequence in pollen cells. The heterologous sequence expressed may encode a protein. Alternatively RNA sequences which do not code for protein (eg ribosomal RNA or anti-sense RNA) may instead be transcribed from the heterologous sequence.

The invention also provides a polynucleotide having the sequence set out in SEQ ID No 1, including derivatives, functional equivalents or parts thereof. The preferred polynucleotide is that shown in SEQ ID No 1 from nucleotides 3144-5396 and more preferably from nucleotides 3430-5349. The most preferred polynucleotide is the promoter in the 3430-5349 bp fragment.

A deposit of genetic material containing the

polynucleotide of SEQ ID No 1 was made at the National Collection of Type Cultures on 7 February 1997 under No NCTC 13013.

The present invention also provides a recombinant nucleotide construct comprising an expression control polynucleotide according to the invention operably linked to a heterologous (preferably protein-encoding) polynucleotide.

Thus, activation of the expression control polynucleotide may drive the expression of the heterologous polynucleotide, enabling production of the encoded protein. Since the expression control polynucleotide is tissue-specific, production of the protein will be limited to those tissues where the expression control polynucleotide is active.

The present invention also provides a recombinant vector containing an expression control polynucleotide or a recombinant nucleotide construct as defined above.

According to the present invention there is also provided a method of producing a recombinant vector, said method comprising ligating an expression control polynucleotide as described above into a suitable vector. A method of producing a transformed cell by transfecting a host cell using said recombinant vector forms another aspect of the invention. Suitable vectors and genetic modifications thereof are well-known in the art.

The present invention also provides a transformed host cell containing a recombinant nucleotide construct or vector as defined above.

The present invention also provides a transgenic organism (for example a transgenic plant) containing a recombinant nucleotide construct or a vector as defined above. The progeny (and seeds) of such transgenic organisms forms a further part of the invention.

The present invention also provides a method for controlling the expression of a protein, said method comprising operably linking a polynucleotide sequence encoding said protein to an expression control polynucleotide of the invention. The method is especially useful for the expression of proteins in pollen. Preferably the protein expressed leads to sterility of the transformed plant.

Thus the invention also provides a method of controlling the expression of a heterologous polynucleotide in pollen, said method comprising operably linking said heterologous polynucleotide to an expression control polynucleotide of the invention.

In one embodiment the promoter for the invertase gene of potato is expressed specifically in pollen to activate expression of any DNA sequences in the pollen of transgenic plants. Below we describe the isolation and characterisation of this promoter and how it has been used to express genes in pollen.

The present invention will now be further described with reference to the Example and accompanying Figures in which:

Figure Legends

Figure 1. Map of sequences detailed in text with restriction enzymes used in their cloning.

Figure 2. whole anther from transgenic potato plant stained for GUS activity (GUS activity indicated by the dark areas).

Figure 3. cross-section of anther as in Figure. 2 showing staining in individual pollen grains (pollen grains appear as dark spots).

Figure 4. RT-PCR analysis showing a product of 374 bp indicating expression from the promoter in (a) floral and bud tissue, and (b) in excised anthers but not in the remainder of the floral tissue.

Example

A potato (*Solanum tuberosum L.*) cv. Saturna genomic library, consisting of a partial Sau3AI digest of genomic DNA cloned into λEMBL3, was plated to yield 1 x 10^5 pfu which were screened with a radiolabelled carrot invertase cDNA fragment generated by reverse transcription-polymerase chain reaction (RT-PCR) using primers derived from a sequence of carrot cDNA (Sturm and Chrispeels, 1990).

The primers were:
Forward Primer: 5'-AACGATCCAAATGGACCA-3' (SEQ ID No 2)
Reverse Primer: 5'-GAAAAAATCAGGACATTCCCA-3' (SEQ ID No 3).

Hybridisation conditions of 5 x SSC at 65°C were utilised with subsequent low stringency washing of filters in 2 x SSC at 65°C. After three rounds of screening two positive clones were obtained plaque pure. DNA was purified from one positive clone, λGF5, which was shown to contain an insert of approximately 23 kb of potato DNA. This cloned potato DNA was

digested with XbaI and SalI, and fragments cloned into pUC19. One subclone, named pGF521, contained 5.4 kb of the potato DNA. A complete DNA sequence of this fragment is presented (SEQ ID No 1). It was determined, by homology to known invertase gene sequences, that the 5.4 kb of potato DNA (Figure 1) carried sequence of two invertase genes with the intergenic region constituting the promoter of the downstream gene. A 2.25 kb HindIII-XbaI fragment (bp 3144-596; Figure 1) comprising the promoter, 3' end of the upstream gene and 5' end of the downstream gene was subcloned into pUC19 to yield plasmid pGF5211 (deposited as NCTC 13013). This fragment was also cloned into pB1101.3 to give plasmid pRM11.2 which was used as a vector for stable plant transformation. In pRM11.2 the fragment is fused to the *uidA* gene from Escherichia coli and when the promoter is active in plants would drive the transcription of this gene to produce the bacteria enzyme β-glucuronidase (GUS). An internal AccI fragment of 1.9 kb (bp 3430-5349; Figure 1) derived from the 2.2 kb fragment was also cloned into pBI101.3 to generate plasmid pRM12.3 which was also used as a vector for stable plant transformation. This fragment was also fused to the *uidA* gene to drive β-glucuronidase synthesis when active.

A series of transgenic lines of potato (cv. Desiree) plants were generated by *Agrobacterium tumefaciens*-mediated transformation using pRM12.3 and pRM11.2 as a vector. Plants derived from the use of pRM12.3 as a vector were passed through one cycle of tuberisation then grown in a controlled environment until flowering occurred. The floral tissues including anthers, sepals, petals and ovules were separately analysed by a GUS histochemical assay performed at two pH values: pH 5 to assay for endogenous enzyme activity (the control)

and pH 7 to detect the activity derived from the *uidA* ene activated by the invertase promoter. A strong blue staining, detected only at pH 7 and thus indicative of bacterial GUS derived from expression of *uidA* driven by the invertase promoter, was observed only in pollen cells (see dark areas of Figures 2 and 3) and in no other tissues of the flower or elsewhere throughout the plant, while in control untransformed plants only a light background of blue staining was observed in pollen cells. Prior to the GUS histochemical analysis an analysis using RT-PCR to detect expression from the native promoter driving its invertase gene had detected expression only in floral and bud tissue with no expression observed in source and sink leaf (Figure 4a), stem, root or tuber. A subsequent RT-PCR analysis detected expression only in the pollen-containing anthers and not elsewhere throughout the flower (Figure 4b). We conclude that the activity of this promoter is restricted to pollen.

The recombinant DNA procedure utilised were as described by Sambrook et al (1989). Plant tissue culture and transformation protocols were as detailed by Hedley (1995). Histochemical assay of GUS was performed as indicated by Jefferson (1987).

The invention describes a promoter sequence demonstrated to be active specifically in pollen of *Solarium tuberosum*. This promoter is likely to be active in pollen of other species of the Solanceae, and may be active in pollen of other plant species including those in which the production of male sterile plants for hybrid production is important eg Lycopersicon esculentum and the Brassicaceae. It is a unique sequence described with this activity in Solanum tuberosum, and for other plants it provides an

alternative to the use of perviously isolated promoters. It has the advantage of its own characteristic activity profile and when used in heterologous species may escape problems such as gene silencing, which can compromise the use of homologous promoters. It has potential use in the genetic engineering of male sterile plants and lines for the restoration of fertility, for the production of proteins in pollen which are toxic to insect and other pests, or for the production of protein of enhanced nutritional value in pollen.

References

Hedley (1995). PhD. Thesis, University of Dundee.

Jefferson (1987). Plant Molecular Biology Reporter 5, 387-405.

Sambrook et al. (1989). Molecular cloning A Laboratory Manual. Second edition. Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sturn & Chrispeels (1990). The Plant Cell 2, 1107-1119.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: EXPRESSION CONTROL POLYNUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10811 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Solanum tubersum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAGCCTAGTT CGACCTGCAG TCAACGGATC TTTATAGCTA CATATATATA AGATTGATCA 60

TTCTTGATAA GCTGGACGTC AGTTGCCTAG AAATATCGAT GTATATATAT TCTAACTAGT 120

AAGAACTATT TTCAAAATTA TGTATACATA CACACACATA CATAATTATG TGGTTCATTT 180

GTGTTAGTTA AAGTTTTAAT ACATATGTAT GTGTGTGTAT GTATTAATAC ACCAAGTAAA 240

CACAATCAAT TCTATTATTC AGTAGTCAGT ATTCATTTTT GAAATGTAAT TAATTTAAAT 300

TTGTGTCTAA AGATAATAAG TCATCAGTCA TAAGTAAAAA CTTTACATTA ATTAAATTTA 360

AACACAGATA ATATTCTATT TTGGAGAACA AAATCGCTCA TGATCAACAA TCGATGACTC 420

AATTTTTAAT TATAAGATAA AACCTCTTGT TTTAGCGAGT ACTAGTTGTT AGCTACTGAG 480

TTAAAAATTA ATTTAAATTC GAAATTAGAT TAATTATTAT GGCAAGACAA TTACAAGGCT 540

AAGGTTTTGT TAAATTTAAG CTTTAATCTA ATTAATAATA CCGTTCTGTT AATGTTCCGA 600

TTCCAAAACG ATAAGAATGT GCAAAAGAGA AAAAGAAACA TGAAATATAT GAAAAAGTTC 660

TTTTAACCTC TATTCTTACA CGTTTTCTCT TTTTCTTTGT ACTTTATATA CTTTTTCAAG 720

AAAATTGGAA AGATTTTGGC CATGGAATTA AGGTGAAAAT TAATTTGTTG GAGGCACCCT 780

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AACATTGGAG ACATTTACAA GTTTGACAAG TATCTTGACC AATGGTAAAA GTCGGTCTTT 10680

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AGTTTTTTTT A 10811

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AACGATCCAA ATGGACCA 18

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Reverse primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAAAAAATCA GGACATTCCC A 21

Claims

1. A pollen cell-specific expression control polynucleotide of an invertase gene, or a derivative, functional equivalent or part of said expression control polynucleotide.

2. An expression control polynucleotide as claimed in Claim 1 which is a promoter.

3. An expression control polynucleotide as claimed in either one of Claims 1 and 2 which comprises a sequence substantially as set out in SEQ ID No 1 or as present in NCTC Deposit No 13013, or a functional equivalent or part thereof.

4. An expression control polynucleotide as claimed in Claim 3 which comprises the sequence of nucleotides 3430-5349 of SEQ ID No 1.

5. A recombinant expression control polynucleotide comprising at least a part of a polynucleotide as claimed in any one of Claims 1 to 4.

6. A recombinant nucleotide construct which comprises an expression control polynucleotide as claimed in any one of Claims 1 to 5 operably linked to a heterologous polynucleotide.

7. A construct as claimed in Claim 6 which is in the form of a vector.

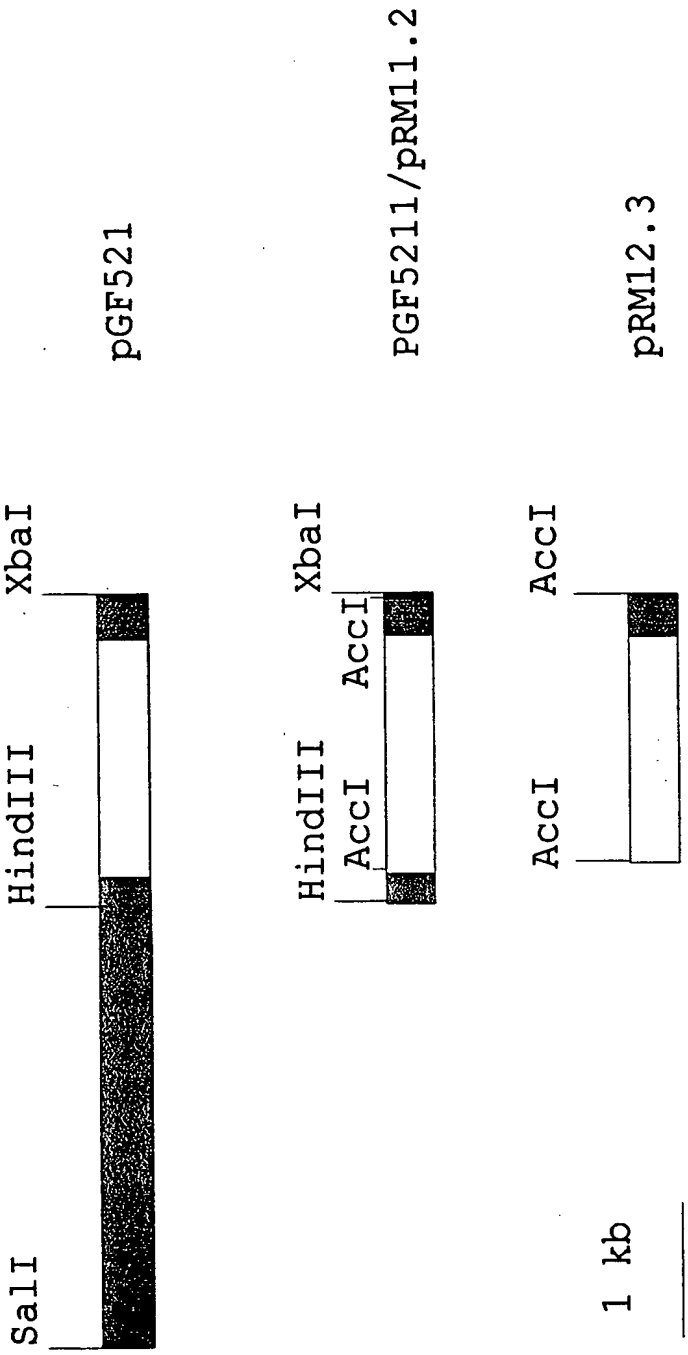
8. A construct as claimed in either one of Claims 6 and 7 wherein said heterologous polynucleotide encodes a protein.

9. A host cell transformed with a construct as claimed in any one of Claims 6 to 8.

10. A transgenic organism transformed with a construct as claimed in any one of Claims 6 to 8.

11. An organism as claimed in Claim 10 which is a plant.

12. An organism as claimed in Claim 11 wherein said expression control polynucleotide is pollen cell-specific and the heterologous polynucleotide operably linked thereto encodes for a protein which causes male sterility of said plant.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HindIII
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AccI
HindIII
AccI
pRM12.3
AccI
AccI
1 kb

Fig. 1

Fig. 2

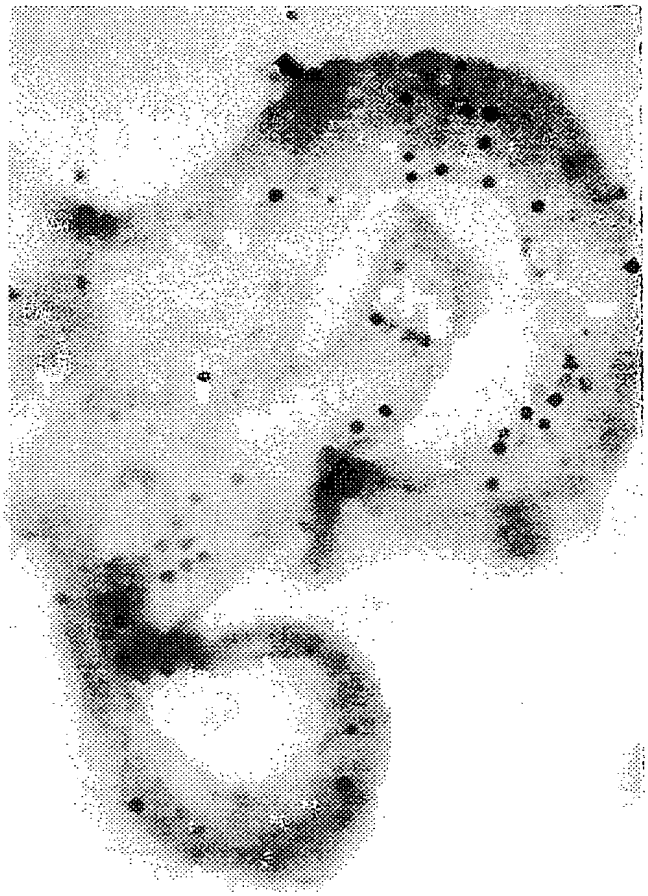

Fig. 3

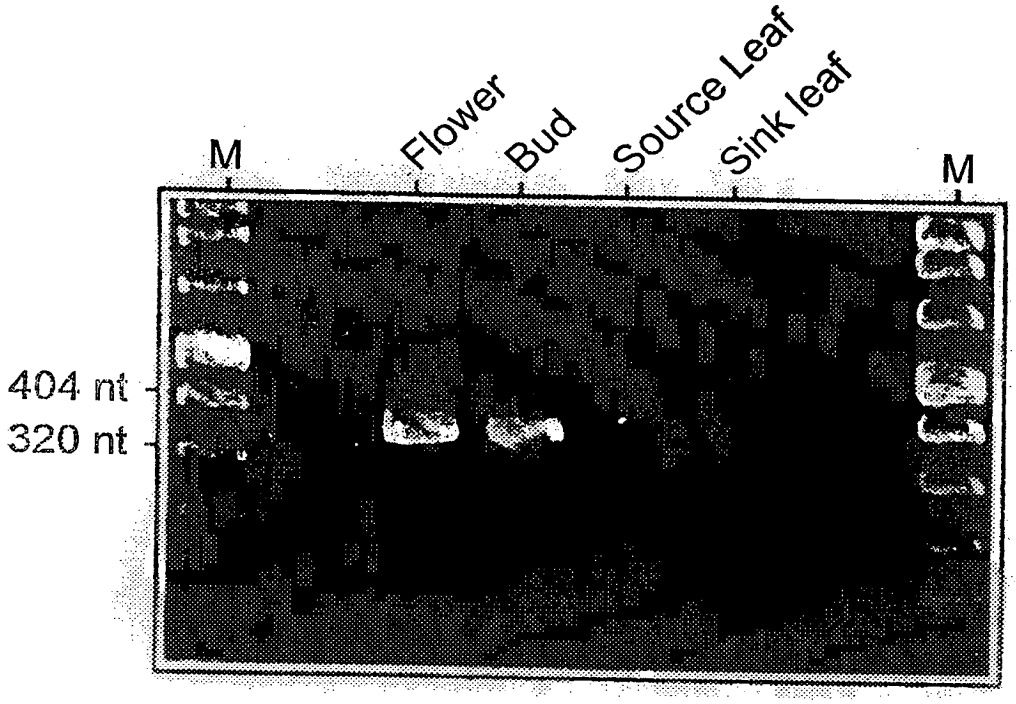

Fig. 4a

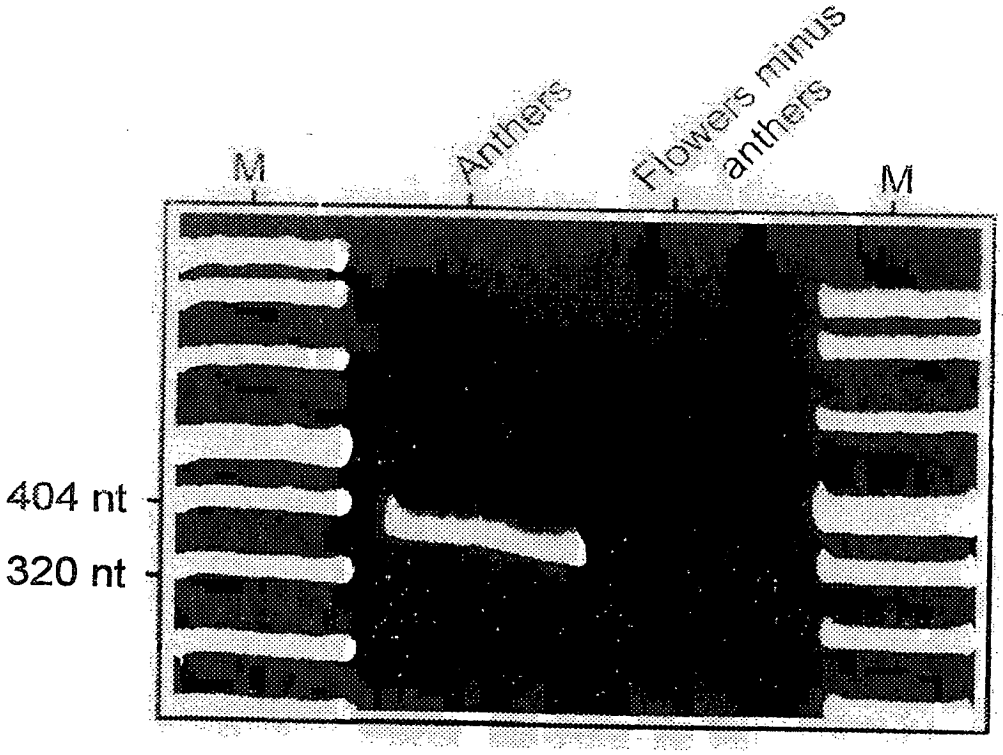

Fig. 4b

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/GB 98/00833

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/56 A01H5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XU J ET AL: "A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: Evidence from a maize invertase gene family." PLANT CELL 8 (7). 1996. 1209-1220. ISSN: 1040-4651, XP002071291 see page 1213, left-hand column, line 11 - line 19 ---	1,2,5-11
Y	MASCARENHAS, J.P.: "Gene activity during pollen development" ANN. REV. PLANT PHYSIOL. PLANT MOL. BIOL, vol. 41, 1990, pages 317-338, XP002071292 see page 329, paragraph 3 - page 331 ---	1,2,5-11
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 1998	27/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/00833

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MEYER R ET AL: "Promoter deletion analysis of potato invertase gene expression." ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY, SWANSEA, WALES, UK, APRIL 11-15, 1994. JOURNAL OF EXPERIMENTAL BOTANY 45 (SUPPL.). 1994. 6. ISSN: 0022-0957, XP002071293 see abstract P2.4 ---	1-12
A	HEDLEY, P.E., ET AL,: "cDNA cloning and expression of a potato (Solanum tuberosum) invertase" PLANT MOLECULAR BIOLOGY, vol. 22, 1993, pages 917-922, XP002071294 see the whole document ---	3,4
A	LORENZ, K., ET AL.: "Structural organization and differential expression of carrot beta-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isozyme" PLANT MOLECULAR BIOLOGY, vol. 28, 1995, pages 189-194, XP002071295 see the whole document & LORENZ, K., ET AL.: "D.carota (Queen Anne's Lace) Inv*Dc2 gene 3432bp" EMBL SEQUENCE DATABASE, ACCESSION NO. X78424, 25 March 1994, see the whole document & STURM A.: "D.carota (Queen Anne's Lace) Inv*Dc1 gene" EMBL SEQUENCE DATABASE, ACCESSION NO. X69321, 23 November 1992, see the whole document ---	3,4
A	HEDLEY, P.E., ET AL.: "Potato (Solanum tuberosum) invertase-encoding cDNAs and their differential expression" GENE, vol. 145, 1994, pages 211-214, XP002071296 see figure 1 ---	3,4

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INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/GB 98/00833

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ALLEN, R.L., ET AL.: "Molecular characterization of one of the maize polygalacturonase gene family members which are expressed during late pollen development" THE PLANT JOURNAL, vol. 3, no. 2, 1993, , pages 261-271, XP002071297 see the whole document	10-12
A	WO 94 01572 A (PIONEER HI BRED INT) 20 January 1994 see page 31	12
A	RAMLOCH-LORENZ, K., E AL.: "Molecular characterization of the gene for carrot cell wall beta-fructosidase" THE PLANT JOURNAL, vol. 4, no. 3, 1993, pages 545-554, XP002071298 see the whole document	1-12

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/GB 98/00833

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9401572 A	20-01-1994	AT 147434 T	15-01-1997
		AU 669384 B	06-06-1996
		AU 4769693 A	31-01-1994
		DE 69307320 D	20-02-1997
		DE 69307320 T	07-08-1997
		DK 651814 T	30-06-1997
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		JP 8501684 T	27-02-1996
		MX 9304114 A	31-05-1994
		NZ 255026 A	26-04-1996
		US 5412085 A	02-05-1995
		US 5545546 A	13-08-1996